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14. ABSTRACT Secondary neuronal damage occurs during the sub-acute phase of spinal cord injury (SCI) and is a major mechanism of permanent neurological dysfunction. The development of new hypotheses focusing on secondary damage is of paramount importance for devising new therapeutics to prevent the morbidity and mortality associated with spinal cord injury in humans. Recently, acidosis-evoked activation of the acid sensing ion channel 1a (ASIC1a) was determined to substantially contribute to secondary neuronal damage following cerebral ischemia. The purpose of this proposal is to determine how ASIC1a contributes to neuron death associated with SCI and whether administration of the ASIC1a inhibitor PcTx1 will significantly reduce neurological deficits following SCI. We will assess both immunohistochemical changes in the lesions as well as behavioral outcomes following injury. In the past year, we have determined that administration of the ASIC1a-inhibiting peptide PcTx1 1 hour after SCI results in an increased number of oligodendrocytes precursors in the injured spinal cord. The presence of the cells could afford better recovery from spinal cord contusion injury and indicates that further studies into the therapeutic potential of ASIC1 inhibitors to limit permanent damage following SCI are warranted. The completion of these aims will provide a foundation to promote further study on the role of acidosis in SCI and elucidate the therapeutic value of ASIC1a inhibition for SCI.					
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INTRODUCTION

Following traumatic injury to the central nervous system, acidosis develops at the injury site due to altered blood flow and abnormal cellular metabolism [1-3]. The acid sensing ion channels (ASICs) are ion channels abundantly expressed in the nervous system [4] and cause neuron death in response to prolonged acidosis [5]. Inhibiting ASIC1a activity, even up to 3 hours AFTER the initial injury, reduces lesion size by up to 50% in mouse models of stroke [6, 7]. ASIC1a is also implicated in pathology due to traumatic brain injury and Multiple Sclerosis, where it affects axonal degeneration [8, 9]. These results suggest that pathological acidosis and inappropriate ASIC1a activation mediate neuronal death throughout the nervous system. Yet, the role of ASIC1a in spinal cord injury (SCI) – another insult in which extracellular pH declines – has not yet been determined. We hypothesize that ASIC1a contributes to neuron death following SCI and that the ASIC1a inhibitor PcTx1 will significantly reduce neurological deficits associated with SCI, even when administered AFTER injury occurrence. To test this hypothesis, we proposed to: (1) Determine how loss of ASIC1a affects outcome from SCI and (2) Determine how pharmacologic inhibition of ASIC1a activity affects neuronal death following SCI in mice. The results from these studies will elucidate the role of the ASIC1 channel in neurological damage following spinal cord injury in rodents. The ultimate goal of this proposal is to determine whether ASIC1 represents a novel new therapeutic target to prevent neurological damage in humans following injury.

BODY

This proposal has two basic aims: (1) to determine how loss of ASIC1a affects neurological outcome following spinal cord injury and (2) to determine how inhibiting ASIC1a activity affects neuronal death following SCI. *Overall completed the major goals of both aims and encountered no unexpected obstacles in completing the experiments as designed.* We have obtained some exciting results as outlined below.

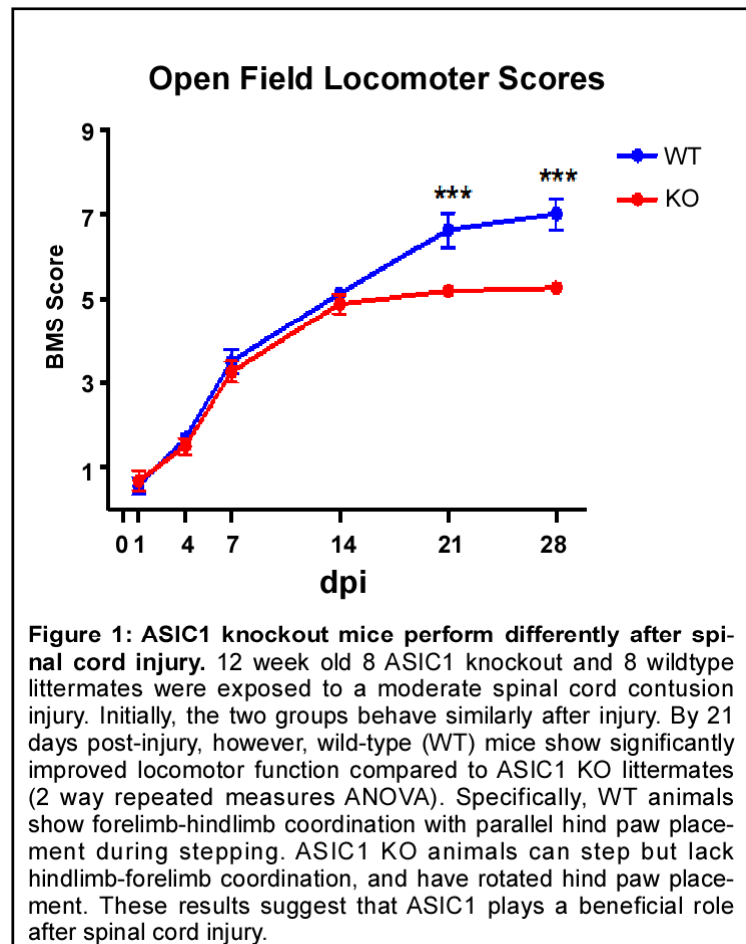
Aim 1: Determine how loss of ASIC1a affects neurological outcome following SCI.

This aim determines how loss of the ASIC1 gene alters spinal cord injury in mice. Specifically, ASIC1 knockout and wild-type littermates will be generated from a colony of knockout mice, subjected to a spinal cord injury, and assessed behaviorally and histologically for any difference related to the genotype of the animal. These experiments will shed light on how ASIC1a contributes to neuronal and glial loss after SCI and whether removal of ASIC1a signaling is neuroprotective and improves functional recovery. Given the strong data published on the detrimental effects of ASIC1a activation in ischemia, the data showing conditions that promote acidification are present after CNS injury, combined with our preliminary data showing ASIC1a is expressed after SCI, we anticipated these experiments would reveal a role of ASIC1a in neuropathology after injury to the spinal cord.

Proposal: Within our statement of work, we proposed to perform a mid-thoracic spinal cord contusion injury on 16 wild-type and 16 ASIC1 knockout animals [10, 11]. The locomotor activity of 8 animals of each genotype would be assessed daily after injury using the Basso Mouse Scale [BMS, 13]. These animals would then be euthanized, tissue isolated, and histochemical analysis performed on spinal cord tissue looking at oligodendrocytes and white-matter sparing. These histochemical techniques allow thorough analysis of lesion severity independent of animal behavior. The other 8 animals of each genotype

would be euthanized at 24 hours after injury, tissue isolated, sectioned and stained for neurons to elucidate early neuronal loss between the two groups. We predict that if ASIC1 plays an important role in spinal cord injury, then mice lacking the ASIC1 gene will show better behavioral recovery and less histochemical markers of lesion severity.

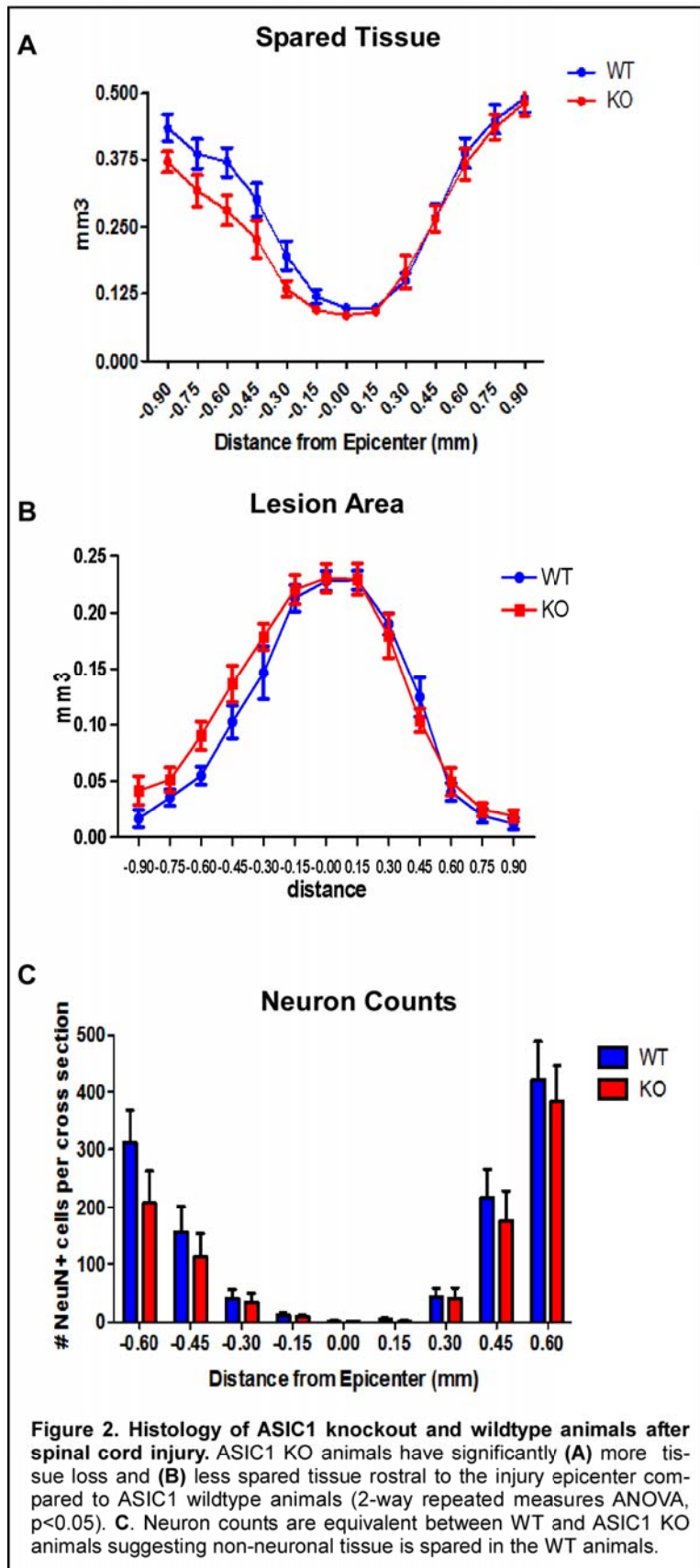
Results: We have completed the major goals of this Aim. Eight 12 week old adult ASIC1a knockout mice and eight wildtype mice were acquired from the colony of C57Bl6/J mice carrying the ASIC1 knockout allele housed and bred by Dr. Askwith. The animals were transferred to Dr. McTigue's Animal Protocol and the Wiseman Hall laboratory two weeks before experiments were performed. One week before the experiment began, mice were handled daily and were allowed to freely explore the open field that was used for locomotor analysis after injury. Mice received a mid-thoracic contusion injury as described and standard post-SCI animal care was performed [see 10,12]. There were no unexpected issues in performing this procedure with the ASIC1 knockout mice. Locomotor recovery of these 11 animals was assessed using the Basso Mouse Scale (BMS; 13) on days 1, 4, 7, 10 and 14. On day 14 post injury, the animals were sacrificed and tissue harvested for histological studies. Analysis of oligodendrocytes and oligodendrocyte precursor cells within the lesion epicenter as well as 0.6 mm rostral and caudal were made and scored. All results were done as outlined within the statement of work.



Results: 8 wildtype animals and 8 ASIC1 knockout littermates were generated and underwent a mid-thoracic spinal cord contusion injury. Locomotor activity was assessed using the BMS scoring system on day 1, 4, 7, 10, 14, 21 and 28 (**Figure 1**). Both groups of animals showed the same level of locomotor dysfunction 1 day after injury and improved similarly until day 14. However, after day 14 the two groups significantly diverge. ASIC1 knockout mice plateau, whereas the wild type mice continued to improve (as indicated by a higher BMS score). These results suggest that the behavioral outcome following spinal cord injury is different between animals lacking the ASIC1 gene and wild-type animals. Further, these results suggest that ASIC1 plays a **beneficial** role following spinal cord injury.

Histological examination of the lesion site supports the behavioral assays (**Figure 2**). Specifically, less tissue sparing is observed in the ASIC1 knockout animals compared to the wildtype littermates. Further, the lesion area is greater in ASIC1 knockout animals. Preliminary data suggest that neuronal numbers are not different between ASIC1 KO and wildtype littermates after injury. This suggests that the difference in lesion size and tissue sparing is due to non-neuronal cells. In support of this hypothesis, we find that oligodendrocyte numbers are likely decreased in ASIC1 knockout animals after injury (**Figure 3**).

Interpretation: Our data strongly suggests that ASIC1 plays a **beneficial** role in spinal cord injury and disruption of the ASIC1 gene in mice exacerbates injury. These results are counter to our original hypothesis as we expected that elimination of the ASIC1 gene would result in less injury and better recovery. Our original hypothesis was based on the role of ASIC1 in stroke and ischemia [6, 7]. Thus, there appears to be a difference in the role of ASIC1 in ischemic neuronal death in the brain versus traumatic induced neuronal death in the spinal cord. This could be due to ASIC1 playing a different role in distinct cell types within the central nervous system. In cerebral ischemia, ASIC1a is localized to the cell bodies and dendrites of neurons [4]. Pathological cerebral acidosis activates these channels which, in turn, kill neurons. In the spinal cord, injury is caused by multiple pathological mechanisms, some of which are distinct from those

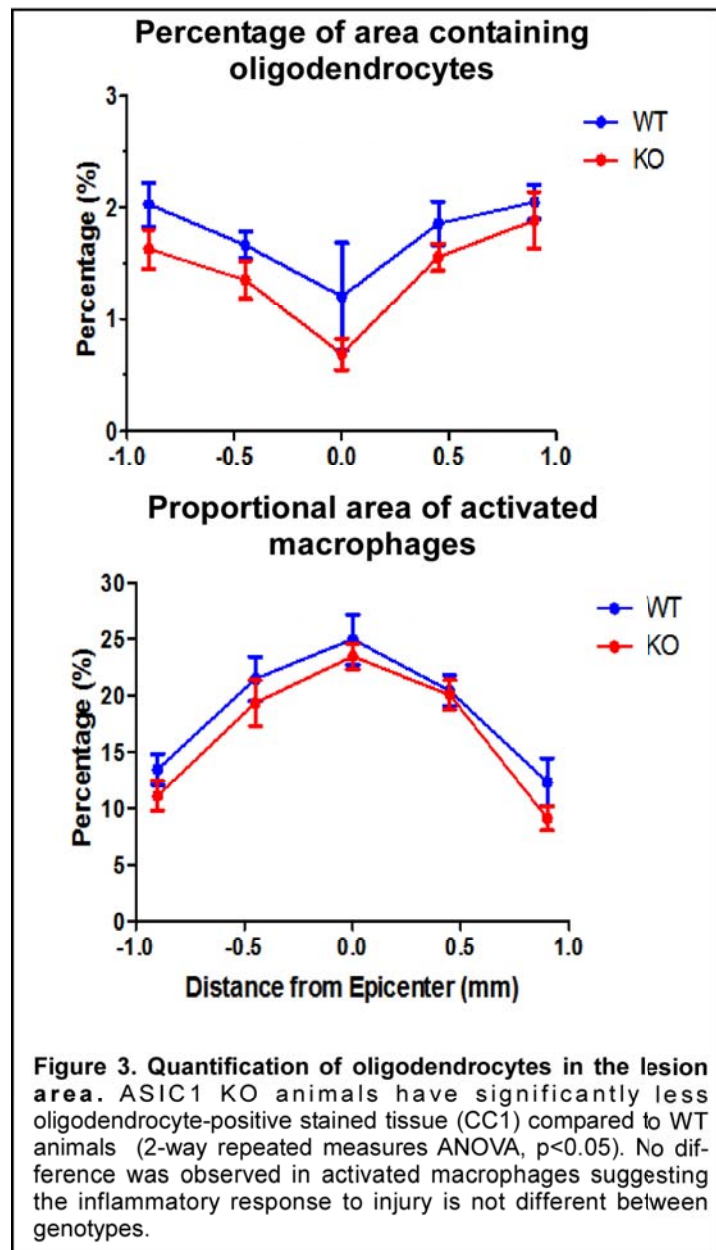


that play a role in cerebral ischemia. Under such conditions, the predominant role for ASIC1a may be within the oligodendrocytes. ASIC1a is present in oligodendrocytes and disruption of ASIC1 has been shown to hinder oligodendrocyte activity [14, 15]. However, it is also possible that genetic disruption of the ASIC1 gene results in compensatory mechanisms within the spinal cord which sensitizes oligodendrocytes to injury. These results are still preliminary and more experiments are necessary to tease apart the contribution of ASIC1 to spinal cord injury. Yet, the implication that ASIC1 could play a neuroprotective role in spinal cord injury is surprising and highly novel. It is essential to investigate this effect to understand the consequences of pharmacological inhibition of ASIC1a during injury.

Aim 2: Determine how inhibiting ASIC1a activity affects neuronal death following SCI.

These experiments will determine if ASIC1a inhibitors (specifically the venom peptide PcTx1) improves anatomical and functional outcomes from SCI, even when given AFTER the injury. These experiments differ from those in Aim 1 in that we are testing whether pharmacological inhibition of ASIC1a can have a therapeutic effect. Psalmotoxin (PcTx1) will be administered at different times after SCI to determine (1) how inhibiting ASIC1a activity affects neuronal survival and (2) how long after SCI can ASIC1a inhibition enhance neuron survival.

Proposal: We propose to purchase 52, 10 week old C57Bl6/J mice from Jackson Labs 2 weeks before performing experiments. Mice will be handled as above the week prior to the experiment. The ASIC1a inhibitor PcTx1 will be purchased from Peptides International, Inc (KY) and tested electrophysiologically prior to use to confirm appropriate activity. Two sets of experiments were proposed. (1) The PcTx1 intrathecal infusion experiment will test whether continuous delivery of PcTx1 over the first 3d post-injury is neuroprotective [7]. BMS testing will be performed on the animals on days 1-3 post-injury, then the 12 animals will be perfused and tissue processing and analysis performed as above. (2) The PcTx1 therapeutic window experiment will test how long



after SCI PcTx1 can be given and still produce neuroprotection [5,7]. Mice will receive an SCI as above. At 1h, 2h, 4h or 8h, mice will be re-anesthetized, the surgery site exposed, and PcTx1 (500ng/ml) or vehicle (aCSF recipe) will be microinjected into the lesion site using a custom-pulled glass pipette as performed previously [11]. The incision will be closed and mice returned to recovery cages. Mice will be followed using the BMS on days 1-3 then perfused and tissue processed and analyzed as above. We will use 40 mice for this study (2 groups x 4 time points x 5/group)

Progress: We have made significant progress on experiment 2 in which PcTx1 or vehicle was injected into the injury site. 30 animals were purchased from Jackson Labs, housed for 2 weeks, and gentled as above. The animals received a mid-thoracic contusion injury as above. 1 group of animals received either vehicle (5 animals) or PcTx1 (5 animals) (500ng/ml) administered to the lesion site. Another group was re-anesthetized 1 hour after surgery and given either vehicle (5 animals) or PcTx1 (500ng/ml) (5 animals) injected intrathecally as described in the statement of work. A third group of animals was re-anesthetized 2 hours after injury and received either vehicle (5 animals) or PcTx1 (500ng/ml) (5 animals). The animals were euthanized after 3 days and tissue was harvested, sectioned, and stained for neurons, oligodendrocytes, and oligodendrocyte precursors.

Results: Administration of PcTx1 to animals following injury was well tolerated and no adverse effects of given the drug were identified. The total neuron count within the lesion area did not reveal any significant differences (**Figure 4**). However, quantification of oligodendrocyte progenitor cells revealed an exciting difference (**Figure 5**). Specifically, the number of oligodendrocyte precursors (as indicated by NG2 positive cells) was increased in the spinal cord of animals treated with PcTx1 1 hour after injury. The effect was observed in the 0.6 mm caudal section of the injury and the trend was also observed at the epicenter, although the data did not reach statistical significance with the “n” available. This effect was not observed in the mice treated with PcTx1 immediately after injury. The presence of the effect when PcTx1 was administered 2 hours after injury is unknown as the preliminary results reported represent an “n” of 1. These results are very exciting as they suggest that the administration of PcTx1 can increase the number of oligodendrocytes at the injury site which could result in more tissue sparing and increased recovery following injury. In an effort to further characterize this effect, we assessed whether the additional cells were present within the white matter or the grey matter of the spinal cord. We determined that the increased oligodendrocyte precursors were present within the grey matter/lesion site, and not the spared white matter (**Figure 6**).

Interpretation: Administration of PcTx1 to the spinal cord 1 hour after spinal cord injury results in increased oligodendrocyte precursors within the grey matter. This effect could impact functional recovery as oligodendrocyte precursor cells give rise to mature oligodendrocytes and the presence of these cells is thought to speed recovery and limit permanent neurological damage. Although we expected PcTx1 administration to affect neuronal damage and death, the acid-sensing ion channel 1a is present on oligodendrocytes and has been proposed to play a role in cellular function and, possibly, death. Further, PcTx1 is known to either inhibit or enhance ASIC1a activity dependent on basal pH. Whether the increased number of cells represents enhanced recruitment or reduced

cell death is unknown. Further, the fact that PcTx1 has no effect when administered immediately after injury suggests that a second event or signal must be present for PcTx1 action. Overall, these experiments support Aim 1 in that oligodendrocytes are the predominant cell type affected by ASIC1 inhibition in spinal cord injury. Future experiments will be performed to investigate the specific mechanism of this PcTx1-mediated effect.

Future Directions: Although the grant has ended, the studies are continuing. We will repeat the studies in Aim 1 with additional animals to complete these studies and ensure that the results are easily replicated. We will test the effects of PcTx1 administration 4 and 8 hours after injury. It is possible that inhibition of ASIC1 can both exacerbate and prevent neuronal damage at distinct times after injury. Exacerbation may come within the earlier time points when acidosis is at its peak. Prevention of neurological injury may occur “later” by enhancing oligodendrocyte action. Thus, administration of PcTx1 at time points more removed from injury may have additional benefit. Future studies will lead to additional insight into these unexpected results.

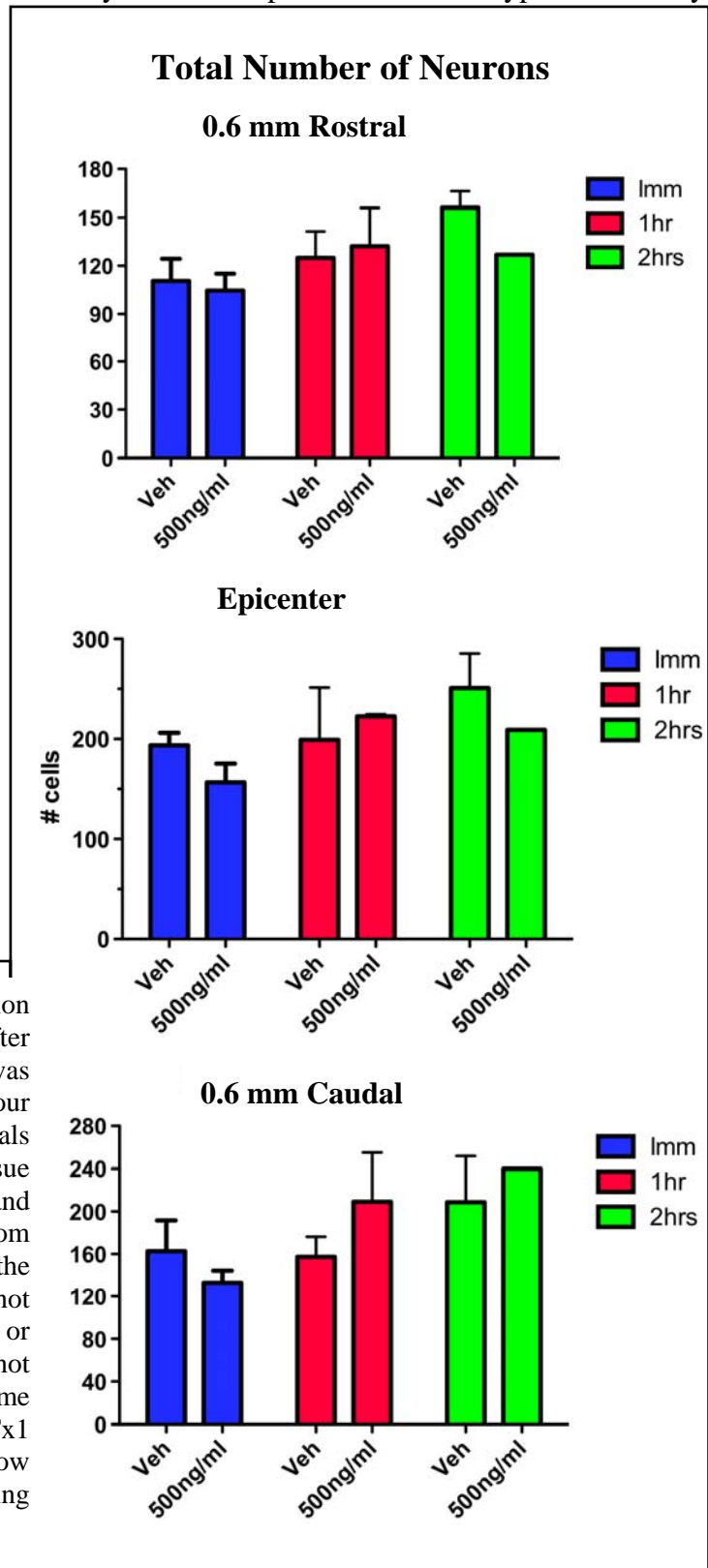
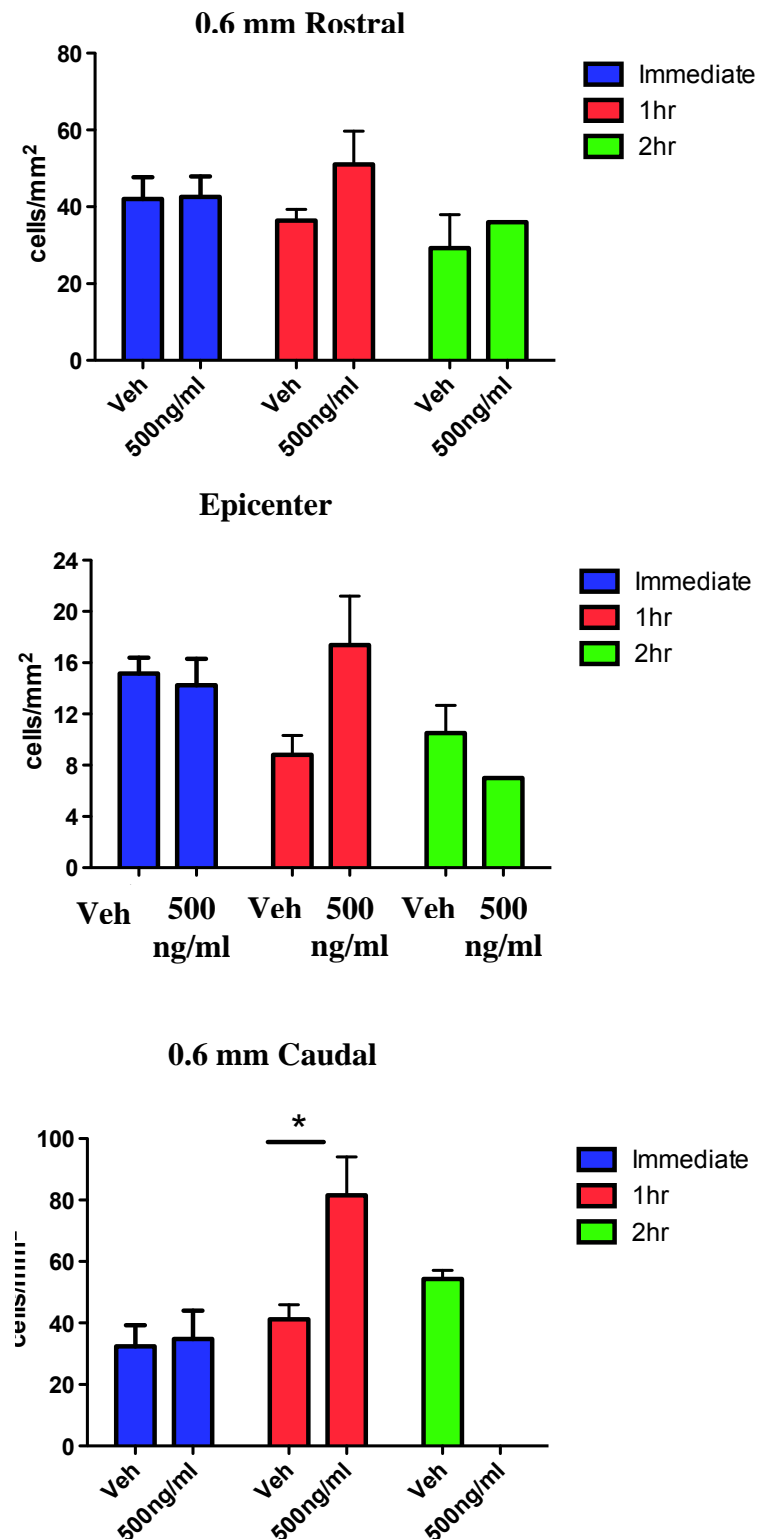
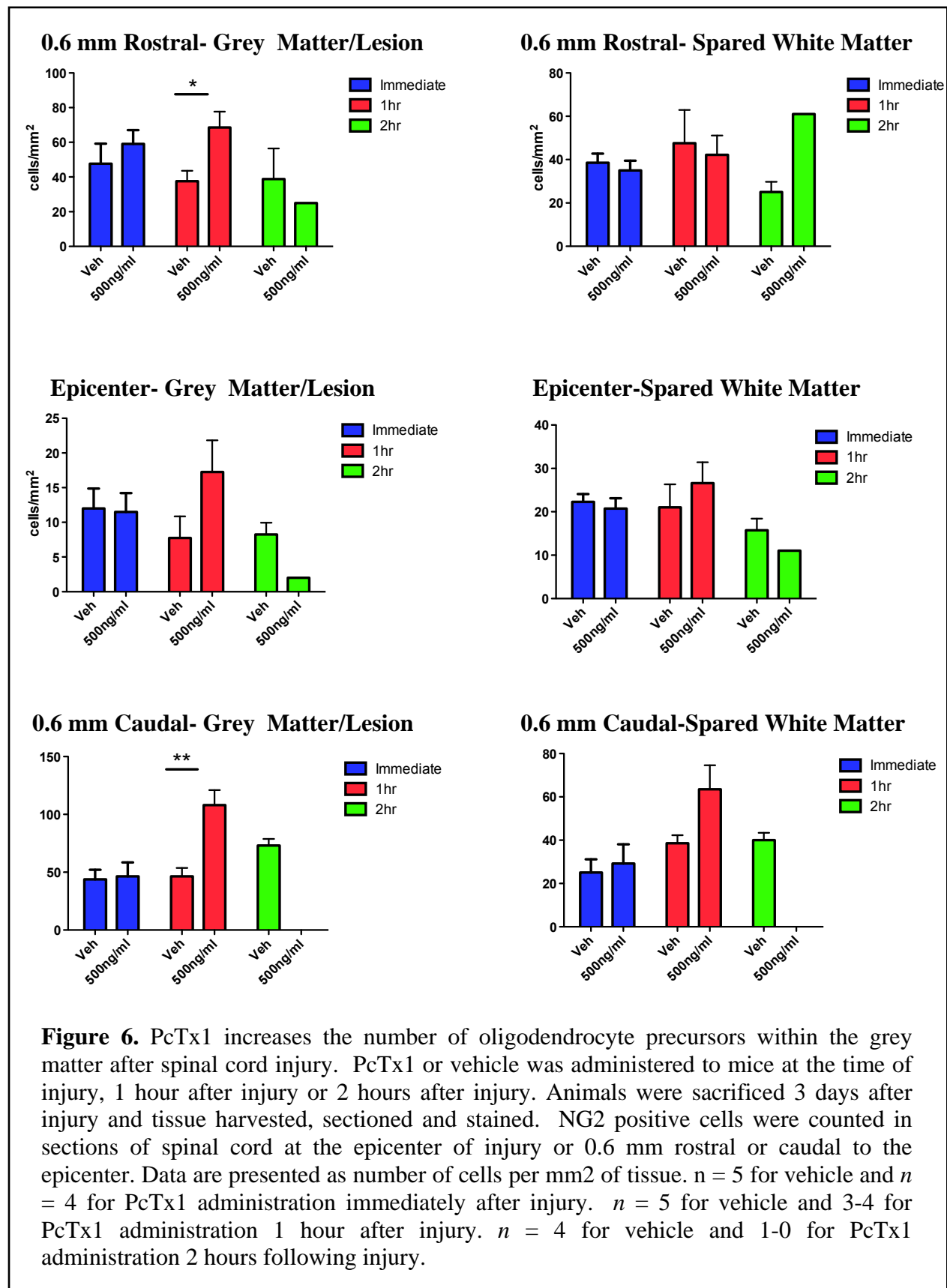


Figure 4. The effect of PcTx1 administration on the number of neurons remaining after spinal cord injury. PcTx1 (500ng/ml) was administered immediately after injury, 1 hour after injury, or 2 hours after injury. Animals were euthanized 3 days after injury, tissue sectioned, and subsequently stained and neurons counted. Neurons were counted from sections taken from the epicenter of the lesion. The total number of neurons was not statistically different at the epicenter or 0.6mm rostral or caudal to the epicenter (not shown). $n = 5-4$ for immediate and 1 hour time point. The $n = 4$ for vehicle and 1 for PcTx1 administration at 2 hours post injury. The low “ n ” reflects an issue with tissue processing and is being rectified.

Figure 5. PcTx1 increases the number of oligodendrocyte precursors after spinal cord injury. PcTx1 or vehicle was administered to mice at the time of injury, 1 hour after injury or 2 hours after injury. Animals were sacrificed 3 days after injury and tissue harvested, sectioned and stained. NG2 positive cells were counted in sections of spinal cord at the epicenter of injury or 0.6 mm rostral or caudal to the epicenter. Data are presented as number of cells per mm² of tissue. *n* = 5 for vehicle and *n* = 4 for PcTx1 administration immediately after injury. *n* = 5 for vehicle and 3-4 for PcTx1 administration 1 hour after injury. *n* = 4 for vehicle and 1-0 for PcTx1 administration 2 hours following injury. The “*n*” of the reported data varies due to processing of the tissue and identification of tissue sections suitable for quantification. Lack of some samples is an expected outcome with studies of spinal cord injury so soon after injury. We are investigating whether the presence of PcTx1 exacerbated these difficulties. “*” indicates statistical significance using an ANOVA analysis





KEY RESEARCH ACCOMPLISHMENTS:

1. Determined that disruption of the ASIC1 gene inhibits behavioral recovery after spinal cord injury in mice
2. Discovered that PcTx1 administration into the spinal cord after injury increases the number of oligodendrocyte precursor cells suggesting potential functional improvement through a novel mechanism.

REPORTABLE OUTCOMES:

This proposal has resulted in salary support for 2 technicians, 1 postdoctoral associate, and 2 principle investigators. The salary support was an important factor in the continued employment of these individuals. This work was presented at the ATACC/MHSRS meeting in Fort Lauderdale, Florida from August 13-16. At this point, we have no other reportable outcomes. However, based on our recent findings more work must be done. Yet, it is expected that this work will produce at least one publication later this year, will be presented at a national meeting within 1 year, and will result in a grant application to the NIH (expected submission date June 2013).

CONCLUSION:

This study tested the novel hypothesis that the acid sensing ion channel 1a (ASIC1a) contributes to neurological damage following spinal cord injury (SCI). The determination that ASIC1a plays a role in SCI would lay the foundation for a new field of study focusing on the role of this ion channel, and pathological acidosis, in SCI mechanisms. Further, these studies would identify ASIC1a, as well as pathological acidosis, as novel therapeutic targets to limit secondary damage following SCI. Previous studies show that inhibiting ASIC1a activity, even hours after the initial injury, causes substantial neuroprotection in rodent models of stroke. Such a result in spinal cord injury would suggest that agents that affect ASIC1a might be administered hours after the initial injury and still prove effective. These experiments represent the first step toward this ultimate goal. From this proposal, we made the exciting observation that genetic deletion of ASIC1 potentiated spinal cord injury. These results suggest that inhibition of ASIC1a during injury or within some time frame after injury may prove detrimental to recovery. Results suggest that oligodendrocytes were primarily affected by loss of ASIC1. Further, we have determined that administration of PcTx1 1 hour after SCI results in an increased number of oligodendrocytes precursors in the injured spinal cord. The presence of the cells could afford better recovery from spinal cord contusion injury. Overall, our results suggest that the role of ASIC1a in spinal cord injury and cerebral ischemia are distinct: with ASIC1a playing a dominant role in oligodendrocytes in the spinal cord. Future studies will definitively determine the role of ASIC1a in spinal cord injury and the therapeutic potential of ASIC1a inhibition to treat SCI.

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